Two novel *Phytophthora* species from the southern tip of Africa

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Abstract

The microbial diversity associated with natural vegetation in the Greater Cape Floristic Region of South Africa is largely unexplored. As part of the Cape Citizen Science programme and independent research, surveys were conducted between 2015 and 2019 to catalogue the diversity of *Phytophthora* species associated with many plant species endemic to this region. Using soil and water baiting techniques, six isolates of the provisionally described *Phytophthora* taxon emzansi were recovered, together with three isolates of an undescribed *Phytophthora* species. In this study, we used both molecular and morphological data to describe these *Phytophthora* species. Isolates of *P. emzansi* sp. nov. and *P. afrocarpa* sp. nov. formed monophyletic lineages within *Phytophthora* Clades 2 and 10, respectively. *Phytophthora emzansi* sp. nov. and *P. capensis* are sister species residing in the *P. citricola* species complex, and both are homothallic. *Phytophthora afrocarpa* sp. nov. is a sister species to *P. gallica,* and both these taxa are sexually sterile. The present study augments our knowledge of the unique *Phytophthora* species associated with the native vegetation of southern Africa.

Keywords Afrotemperate forests, fynbos, Greater Cape Floristic Region, multi-gene phylogeny, Western Cape Province

Introduction

The plant diversity of South Africa is known to be among the richest in the world. For example, the Greater Cape Floristic Region contains two globally recognized biodiversity hotspots, a sub-region generally referred to as the Cape Floristic Region (CFR) and the Succulent Karoo (Myers et al. 2000; Snijman 2013). Even though the CFR accounts for less than 5% of the land area in South Africa (90,000 km^{-2}), it contains 44 % of the vascular plant species in southern Africa, of which 70 % are endemic (Goldblatt and Manning 2002). Much of this vegetation is characterized as fynbos scrubland (Born et al. 2007). Another notable, but distinct vegetation type is the afrotemperate forests. The largest complex of these forests is situated in the Southern Cape, which receives year-round rainfall and contains a few endemic coniferous species, including *Podocarpus latifolius* and *Afrocarpus falcatus* (Barker et al. 2004). This endemic flora of southern Africa is threatened by various native and invasive pests and pathogens, including species belonging to the plant pathogenic oomycete genus *Phytophthora* (Hulbert et al. 2019; Bezuidenhout et al. 2010; van Wyk 1973; von Broembsen 1984; von Broembsen et al. 1986).

Many *Phytophthora* species have been reported from the CFR and southern afrotemperate forests (Bezuidenhout et al. 2010; von Broembsen 1984; van Wyk 1973; Lübbe and Mostert 1991). Among these, the invasive pathogen *P. cinnamomi* is of particular concern, because it causes root and collar rot of many endemic plant species (von Broembsen 1984; von Broembsen and Kruger 1985; von Broembsen et al. 1986). While cataloguing the diversity of *Phytophthora* species associated with native *Agathosma* species in the Western Cape Province of South Africa, Bezuidenhout et al. (2010) identified two novel species, *P. capensis* and *P.* taxon emzansi. Due to the low recovery and high degree of morphological variation between the isolates of *P.* taxon emzansi (STE-U 6269 and STE-U 6272), the authors chose to assign a provisional name to the organism. Later, *P.* taxon emzansi was reported from the KwaZulu-Natal Province of South Africa, but its taxonomic status was not considered (Oh et al. 2013).

As a part of the Cape Citizen Science programme (https://citsci.co.za/) and independent research, surveys were conducted between 2015 and 2019 in the Southern Cape region of South Africa. During

these surveys, six isolates of *P.* taxon emzansi, along with three isolates of an undescribed *Phytophthora* species were recovered. In the present study, DNA sequence comparisons and morphological observations were used to revise the taxonomy of *P.* taxon emzansi and describe the newly recovered *Phytophthora* species.

Materials and methods

Collection of samples and baiting

Samples were collected at different time points throughout the year between 2015 - 2019. Soil and fine roots were collected from the rhizosphere of plants after removing the 2-3 cm of topsoil and plant litter. As part of Cape Citizen Science, 665 samples were collected with or submitted by citizens and an additional 75 trees were independently sampled in five afrotemperate forest sites throughout the Southern Cape region (JM Hulbert unpublished). Water samples were also collected from streams or irrigation ponds on a few occasions (Hulbert et al. 2019).

All soil and water samples were baited separately in the laboratory. For each soil baiting, approximately 300 g of soil was placed in a plastic tray containing 1 L of non-sterile distilled water. The floating plant litter was discarded and the baits were placed on the surface of the water. A litre of each water sample was also baited in a plastic tray where the baits were floated on the surface of the water (Oh et al. 2013). Leaves of *Rhododendron indicum*, *Hedera helix*, *Leucospermum rodolentum*, *Quercus robur*, and petals of *Rosa* sp. served as baits.

The baits were monitored regularly for lesions over 10 days. Symptomatic baits with lesions were plated onto the *Phytophthora* selective medium, NARPH (50 mg Nystatin, 200 mg Ampicillin, 10 mg Rifampicin, 25 mg Pentachloronitrobenzene, and 50 mg Hymexazol per 1 L of deionized water and 15 g cornmeal agar as described by Hüberli et al. (2000)). Pure cultures were maintained on half-strength Potato Dextrose Agar (PDA; 19.5 g PDA powder, Merck, South Africa; 7 g Difco agar; 1 L of deionized water) and as agar blocks suspended in sterile deionized water in glass vials.

Isolates of *P.* taxon emzansi (STE-U 6269 and STE-U 6272) from the study of Bezuidenhout et al. (2010) were retrieved from the microbial culture collection of the Department of Plant Pathology, Stellenbosch University and these isolates were included in the molecular analysis of the current study.

DNA isolation, amplification and sequencing

A ZR Fungal/Bacterial DNA MiniPrep™ (Zymo Research, USA) kit was used to extract the total genomic DNA from ten-day-old cultures of eight isolates of *P.* taxon emzansi and three isolates of the undescribed *Phytophthora* species, following the manufacturer's protocols. The complete ITS region and partial cytochrome oxidase I (*COX1*), β-tubulin (*BT*), and heat shock protein 90 (*HSP90*) genes were amplified.

Each 25 µl PCR reaction included: 2.5 µl of 10× KAPPA Taq Buffer A (Kapa Biosystems, Cape Town, South Africa), 0.5 µl of 0.1 mM dNTPs (Promega, MI), 1 µl each of forward and reverse primers, 0.2 µl of KAPA Taq DNA Polymerase (Kapa Biosystems, Cape Town, South Africa), 18.8 µl PCR grade water, and 1 µl of DNA template. PCR amplifications were conducted with an initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec; annealing temperatures 55 ºC for 30 sec (ITS, ITS6 / ITS4; Cooke et al. 2000; White et al. 1990), 60 ºC for 30 sec (*BT*, Btub_F1A / BTub_R1; Blair et al. 2008; Kroon et al. 2004), 56 ºC for 60 sec (*COX1*, FM84 / FM83; Martin and Tooley 2003), and 62 ºC for 30sec (*HSP90*, HSP90_F1 / HSP90_R2; Blair et al. 2008); 72 °C for 1 min; and final elongation at 72 °C for 7 min.

The amplicons were sequenced at the DNA sequencing facility of the University of Pretoria and assembled with CLC Main Workbench 20.0.2 (QIAGEN, Aarhus). The BLAST algorithm (Altschul et al. 1990) available from the NCBI GenBank was used for the initial identification of the sequences. GenBank accession numbers for sequences generated in this study are listed in Table 1.

Table 1 NCBI GenBank accession numbers for *Phytophthora* species used for phylogenetic analyses. Accession numbers in bold font emerged from the present study

Taxa	Isolate No.	Clade	Gene regions			
			ITS	*BT*	*COX1*	*HSP90*
P. emzansi	CBS 147464	2	**MT762301**	**MT762327**	**MT762309**	**MT762318**
P. emzansi	CBS 147465	2	**MT762302**	**MT762328**	**MT762310**	**MT762319**
P. emzansi	CBS 147466	2	**MT762303**	**MT762329**	**MT762311**	**MT762320**
P. emzansi	CMW54569	2	**MT762304**	**MT762330**	**MT762312**	**MT762321**
P. emzansi	CMW54577	2	**MT762305**	**MT762331**	**MT762313**	**MT762322**
P. emzansi	CMW50975	2	**MN545898**	**MT762332**	**MT762314**	**MT762323**
P. emzansi	STE-U 6269	2	GU191228	GU191270	GU191317	**MT762299**
P. emzansi	STE-U 6272	2	GU191220	GU191269	GU191316	**MT762300**
P. capensis	CPHST BL 10	2	MG865466	MH136862	MH493914	MK020282
P. acerina	CPHST BL 114	2	MG518642	MH136845	MH493901	KX250716
P. plurivora	CPHST BL 74	2	MG865568	MH136959	MH494001	MK020372
P. caryae	NJB2013-AF-08	2	KJ631538	KU696638	KP749393	-
P. pini	CPHST BL 48	2	MG865565	MH136957	MH493998	MK020369
P. citricola	CPHST BL 34	2	MG865475	MH136871	KX250748	MK020288
P. pachypleura	CPHST BL 146	2	MG865558	MH136948	MH493991	KX250793
P. multivora	CPHST BL 104	2	MG865546	MH136939	KX250776	KX250779
P. citrophthora	CPHST BL 60	2	MG865476	MH136872	JN605909	MK020289
P. occultans	CPHST BL 163	2	MG865555	MH477753	MH493990	MK020359
P. himalsilva	CPHST BL 102	2	MG865507	MH136901	KX250573	KX250576
P. terminalis	CPHST BL 164	2	MG865592	MH136984	MH494018	MK020395
P. botryosa	CPHST BL 132	2	MK496516	MH136855	MH493910	KX250541
P. colocasiae	CPHST BL 173	2	MG865479	MH136875	KX227473	-
P. meadii	CPHST BL 81	2	MG865529	MH136924	KX250594	KX250597
P. mekongensis	PF6a2	2	KC875838	KT366920	-	-
P. capsici	CPHST BL 33G	2	MG865467	MH136863	MH493915	MK020283
P. mexicana	CPHST BL 24	2	MG865540	MH136933	KX250671	KX250674
P. glovera	CPHST BL 36	2	MG865500	MH136895	MH493943	KX250653
P. amaranthi	CPHST BL 174	2	MG783373	MH477739	KJ179949	-
P. tropicalis	CPHST BL 58	2	MG865596	MH136987	MH494022	MK020399
P. siskiyouensis	CPHST BL 56	2	MG865586	MH136978	KX250678	KX250681
P. mengei	CPHST BL 31	2	MG865539	MH136932	KX250657	MK020344
P. oleae	Po1a	2	KY982930	MF083569	-	-
P. multivesiculata	CPHST BL 50G	2	MG865544	MH136937	MH493982	EU080069
P. bisheria	CPHST BL 6	2	MG783381	MH136851	MH493908	-
P. frigida	CPHST BL 39G	2	MG865496	MH136892	MH493940	KX250919
P. elongata	CPHST BL 62	2	MG865485	MH136881	MH493932	MK020301
P. afrocarpa	CBS 147467	10	**MT762306**	**MT762333**	**MT762315**	**MT762324**
P. afrocarpa	CBS 147590	10	**MT762307**	**MT762334**	**MT762316**	**MT762325**
P. afrocarpa	CBS 147468	10	**MT762308**	**MT762335**	**MT762317**	**MT762326**
P. gallica	CPHST BL 35	10	MG865497	MH136893	MH493941	MK020308

P. intercalaris	45B7	10	KT163268	-	MF543336	KX252614
P. boehmeriae	CPHST BL 32G	10	MG783382	MH136852	DQ361135	MK020276
P. gondwanense	W1858	10	KP070695	-	KX252604	KX252607
P. kernoviae	CPHST BL 91	10	MG865521	MH136915	HM534914	MK020328
P. morindae	CPHST BL 49G	10	MG865543	MH136936	KX252634	KX252637
P. richardiae	CPHST BL 76	10	MK496521	MH136974	MH494010	EU080641
P. macrochlamydospora	CPHST BL 71	10	MG865528	MH136923	MH493968	MK020335
P. quininea	CPHST BL 54G	10	MG865580	MH136972	MH494009	EU079805
P. captiosa	CPHST BL 11	10	MG865469	MH136865	MG543042	MG543029
P. fallax	CPHST BL 63	10	MG865489	MH136885	MG543044	MG543031
P. constricta	CPHST BL 61	10	MG865480	MH136876	MG543049	MG543035
P. cacuminis	U40	10	MG542997	MG543045	MG543010	MG543032
P. alticola	CBS141718	4	KX247599	KX247589	KX247598	KX247583
P. nicotianae	CPHST BL 44	1	MG865550	MH136943	MH493986	MK020353
P. cinnamomi	CPHST BL 12	7	MG865473	MH136869	MH493920	KX251815

Phylogenetic analyses

Initial identification of the sequences showed eight isolates of *P.* taxon emzansi and three isolates of the undescribed *Phytophthora* species resided in *Phytophthora* Clades 2 and 10, respectively. Therefore, for phylogenetic analyses, separate datasets for each of these clades were prepared for four gene regions (ITS, *BT*, *COX1* and *HSP90*). Each of these datasets included sequences generated in this study and those from the ex-type isolates (where available) of all formally described *Phytophthora* species listed in the database IDPhy (http://idtools.org/id/phytophthora/index.php, Abad et al. 2019). All the datasets were aligned using MUSCLE 3.8.31 (Edgar 2004) and manually adjusted where necessary using Mesquite 3.61 (Maddison and Maddison 2019). Maximum likelihood analyses and model testing for single gene and concatenated datasets were completed using IQ-TREE 1.6.8 (Nguyen et al. 2015). Bayesian analyses of all sequence datasets were done using Mr. Bayes 3.2.7a (Huelsenbeck and Ronquist 2001). The alignments and trees were deposited in TreeBASE (Study ID S26766).

Colony morphology and growth in culture

The colony morphology of six isolates of *P.* taxon emzansi and three isolates of the undescribed *Phytophthora* species obtained during the current study were described following the suggested system of Erwin and Ribeiro (1996). Inoculum plugs (5 mm diam.) were taken from the margins of eight-day-

old cultures growing on half-strength PDA at 20 ºC in the dark and transferred to four microbial culture media: 10 % Carrot Agar (CA; 10 ml fresh carrot juice; 15 g Difco agar, Becton, Dickinson and Company, Sparks, USA; 1 L of deionized water), 2 % Malt Extract Agar (MEA; 20 g malt extract, Merck, South Africa; 15 g Difco agar and 1 L of deionized water), 10 % clarified V8-Agar (V8A; 10 ml clarified V8 juice, Campbell Soup Company USA; 15g Difco Agar; 1 L of deionized water), and half strength PDA. The colony morphologies were evaluated and described after the inoculated plates were incubated at 20 ºC for seven days in the dark.

Growth rates were determined for all isolates of both species obtained during the current study, with *Phytophthora cinnamomi* (CMW48774) included for comparative purposes. Isolates were initially sub-cultured on V8A and incubated for 24 h at 20 °C in the dark. Thereafter, five replicate plates per isolate were incubated at 4, 10, 15, 20, 25, 30, and 35 ºC (± 0.5 ºC). Colony diameters were measured daily from the third to the seventh day of incubation. For viability assessment, all cultures incubated at 4, 10 and 35 °C were returned to 20 °C in the dark to check for the resumption of growth after 48 h.

Morphology of sporangia and gametangia

The morphology of sporangia and gametangia were studied for six isolates of *P.* taxon emzansi and the three isolates of the undescribed *Phytophthora* species recovered in this study. To induce sporangial production, agar blocks (~ 1 cm × 1 cm) were cut from the actively growing edges of eight-day-old colonies growing on half-strength PDA. Excised agar blocks from each isolate were transferred to individual Petri dishes with deionized water. The water was replaced after 2, 4 and 6 h respectively. After the last water change, 1 ml of 10 % unsterilized soil extract was added and the plates were incubated overnight at 21 ºC in darkness. After 24–48 h, measurements were made for 50 randomly selected sporangia for each isolate of the two *Phytophthora* species. Photographic images were captured using an AxioCam ICc5 camera attached to a ZEISS Axioskop50.

For the *P.* taxon emzansi isolates, gametangia were readily produced after 9 -12 days on half-strength PDA and V8A incubated at 20 °C in dark. After 12 days, dimensions of 25 randomly selected mature oogonia, oospores and antheridia were measured at 400× magnification. The oospore wall index was calculated as the ratio between the volume of the oospore wall and the volume of the whole oospore

(Dick 1990). In contrast, the isolates of the undescribed Clade 10 *Phytophthora* species failed to produce any gametangia, despite being carefully observed for 30 days. In an attempt to induce gametangia in these isolates, they were crossed with *Phytophthora cinnamomi* A1 (CMW29606) and A2 (CMW29597) mating type strains on 10% V8A amended with 0.05 g β - sitosterol. All Petri plates were incubated at 20 °C in dark and evaluated again after 21 days.

Results

Phylogenetic analyses

Phylogenies of *Phytophthora* Clade 2 species were mostly congruent with trees published by Bezuidenhout et al. (2010). In the present study, trees from phylogenetic analyses of individual genes (Fig. S1) and concatenated (Fig. 1) datasets for all four gene regions had similar topologies. The six isolates of *P.* taxon emzansi recovered in this study formed a monophyletic clade together with those from the study by Bezuidenhout et al. (2010) (STE-U 6269 and STE-U 6272). Irrespective of the datasets, all the clades were supported by high bootstrap values and posterior probabilities (Fig. 1 and S1). As previously reported by Bezuidenhout et al. (2010), *P.* taxon emzansi is as a sister species to *P. capensis* (Fig. 1, S1), residing in the *P. citricola* complex.

In the phylogeny for the three isolates of the *Phytophthora* species residing in Clade 10; the topology of trees recovered from single genes (Fig. S2) and concatenated datasets for the four gene regions were similar (Fig. 2). In these phylogenies, all three isolates emerged as an undescribed *Phytophthora* species forming a monophyletic clade with the Clade 10b species, *P. boehmeriae*, *P. gallica*, *P. gondwanense*, *P. intercalaris*, *P. kernoviae*, and *P. morindae* (Abad et al. 2019). This novel *Phytophthora* species was the sister taxon to *P. gallica* (Fig. 2, S2).

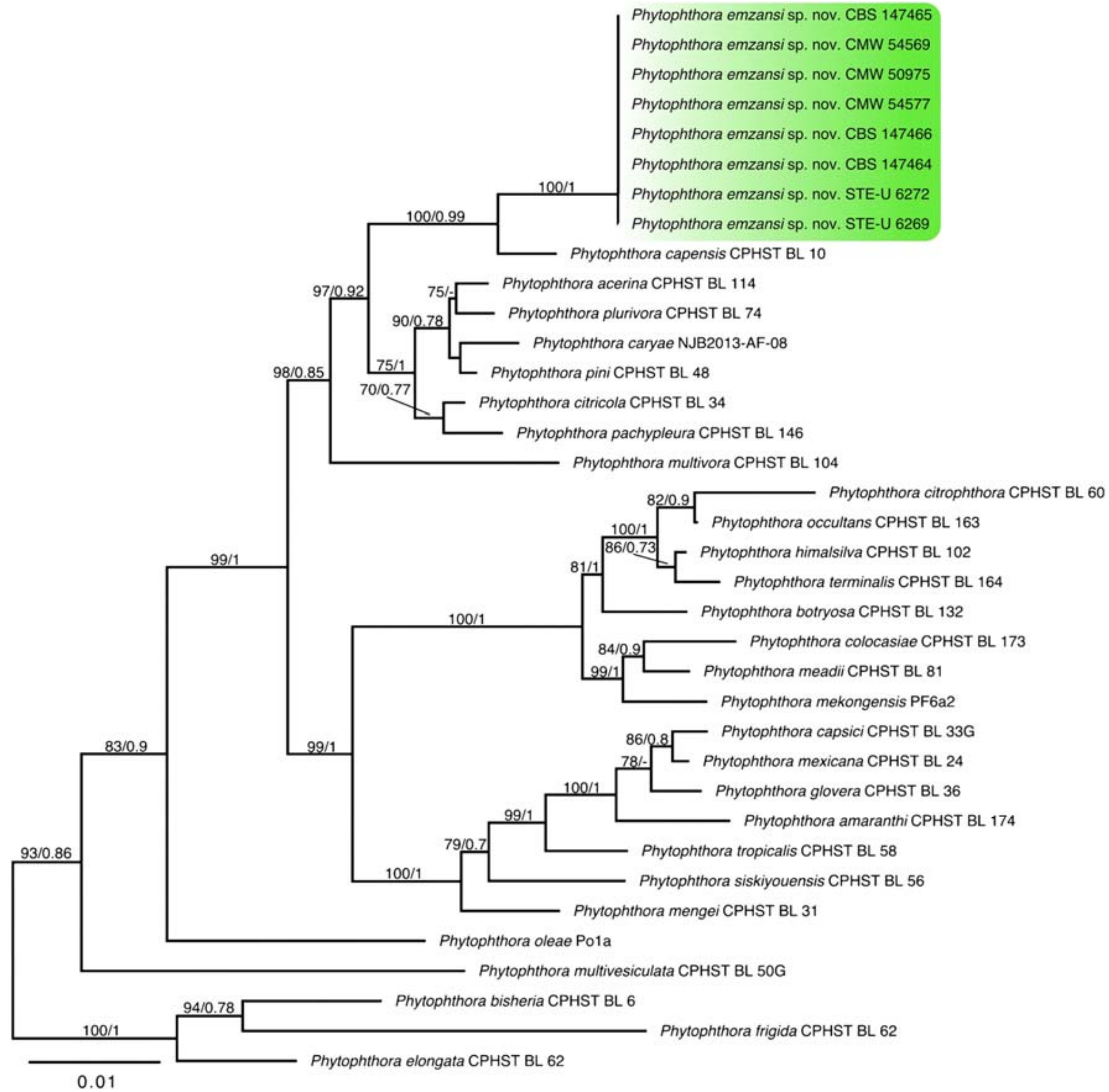

Fig. 1 Maximum likelihood phylogeny using concatenated dataset for ITS Clade 2 *Phytophthora* species. The monophyletic clade of *Phytophthora emzansi* sp. nov. is highlighted in green. *Phytophthora cinnamomi* CPHST BL 12 was used as an outgroup taxon (not shown). Numbers on the branches are bootstrap values (≥ 70) / posterior probabilities (≥ 0.7)

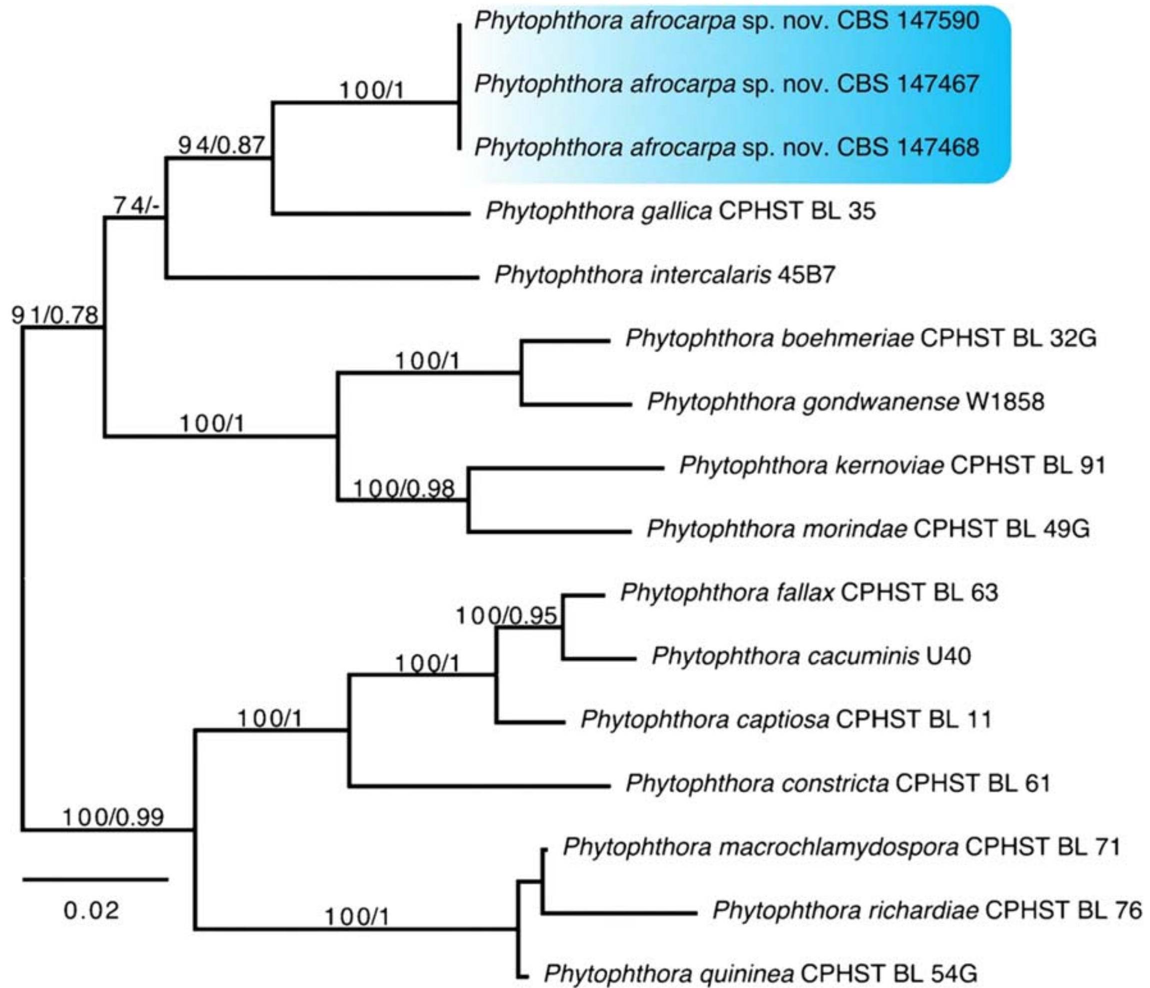

Fig. 2 Maximum likelihood phylogeny using concatenated dataset for ITS Clade 10 *Phytophthora* species. The monophyletic clade of *Phytophthora afrocarpa* sp. nov. is highlighted in blue. *Phytophthora alticola* CBS141718 and *P. nicotianae* CPHST BL 44 were used as outgroup taxa (not shown). Numbers on the branches are bootstrap values (≥ 70) / posterior probabilities (≥ 0.7)

Taxonomy

***Phytophthora emzansi* T. Bose, T. Paap, and J.M. Hulbert sp. nov.**

Mycobank. MB838530

Etymology. Name derived from the Zulu word for south or from the south as previously noted by Bezuidenhout et al. (2010).

Type. SOUTH AFRICA, Knysna Forest, Western Cape Province, from rhizosphere soil of *Afrocarpus falcatus* (-33.93149201, 22.62277904), November 2017, leg. JM Hulbert type PREM63081, ex-type culture PPRI 28451 = CMW 54354 = CBS 147464. GenBank: ITS: MT762301, *BT*: MT762327, *COX1*: MT762309, *HSP90*: MT762318.

Description. Sporangia shape predominantly ovoid (72%; Fig. 3a), occasionally elongate ovoid, pyriform or obpyriform (Fig. 3b); mostly semi-papillate (86%; Fig. 3a), non-papillate (10%; Fig. 3c), or rarely papillate (4%; Fig. 3d), often with displaced papilla (Fig. 3e); persistent, abundantly produced within 48 h of adding non-sterile soil extract; predominantly solitary, terminal in position, occasionally in loose sympodia (Fig. 3f). Sporangia from six isolates measured (25.0) 26.7 ± 1.01 (63.8) × (17.5) 23.9 ± 0.6 (41.5) µm, exit pores ranging from (3.8) 5.6 ± 0.21 (11.46) µm, length to breadth ratio 1.4 ± 0.02. Encysted zoospores ranging from 8.2 – 17.7 µm. Sporangiophores simple or rarely branched, attachment usually basal (Fig. 3a), sometimes lateral. Chlamydospores absent. Homothallic; Oogonia (18.75) 35.4 ± 1.05 (49.93) µm in diam. (Figs. 3g, h), frequently with a tapering oogonial stalk (Fig. 3h); abortive oogonia common (39%). Oospores usually plerotic (80%; Figs. 3g, h) or aplerotic (20 %), with a smooth surface (Figs. 3g, h), on maturation light golden brown in colour (Figs. 3g, h). Oospores (18.13) 31.1 ± 0.89 (46.75) µm in diam. Oospore wall thickness is highly variable (0.6) 2.0 ± 0.13 (5.0) µm; oospore wall index ranging from (0.28) 0.38 ± 0.11 (0.45) µm. Antheridia amphigynous, measuring (6.25) 14.2 ± 0.8 (24.5) × (7.8) 16.1 ± 0.77 (24.9) µm (Figs. 3g, h). Hyphae coenocytic, highly branched, lateral branches often short and stubby with terminal or intercalary hyphal swellings measuring (3.8) 13.4 ± 0.81 (28.54) µm (Fig. 3i), often forming coralloid hyphae (Fig. 3i).

Colony morphology and growth. White, filamentous hyphae, slightly raised, cottony with no distinct pattern on CA, V8A, PDA, CMA and MEA (Fig. 4a-e). Colony lax and immersed on CMA and MEA (Fig. 4d-e). Growth extremely slow on MEA after 5 days at 20 °C in darkness (Fig. 4e). Optimum growth temperature for all six isolates on V8A was 20 °C (10.22 ± 0.49 mm/day; Fig. 5). The minimum and maximum temperatures for growth were 4 °C (0.22 ± 0.02 mm/day; Fig. 5) and 30 ºC (0.25 ± 0.02 mm/day; Fig. 5), respectively. Plates incubated at 35 °C showed no growth when tested for viability after the growth study, indicating that this temperature was lethal.

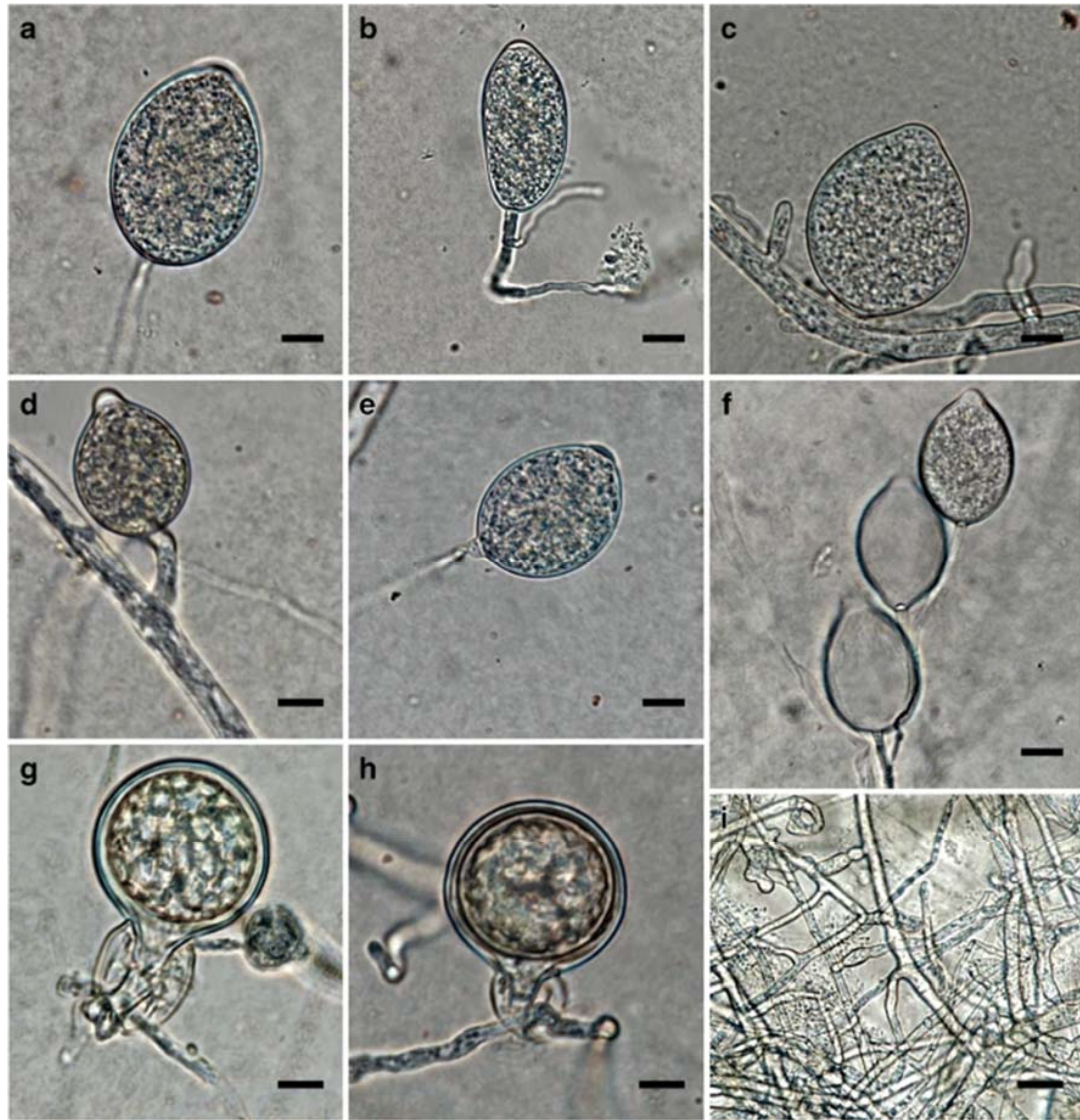

Fig. 3 Morphology of *Phytophthora emzansi* sp. nov. recovered in this study. **a** ovoid semi-papillate sporangium with basal sporangiophore. **b** obpyriform sporangium. **c** non-papillate sporangium. **d** papillate sporangium with short stocky sporangiophore. **e** ovate sporangium with basal off-centred sporangiophore. **f** external unilateral sporangial proliferation. **g, h** mature oogonium with plerotic oospore with amphigynous antheridium. **i** hyphal swellings. Bars 10 µm

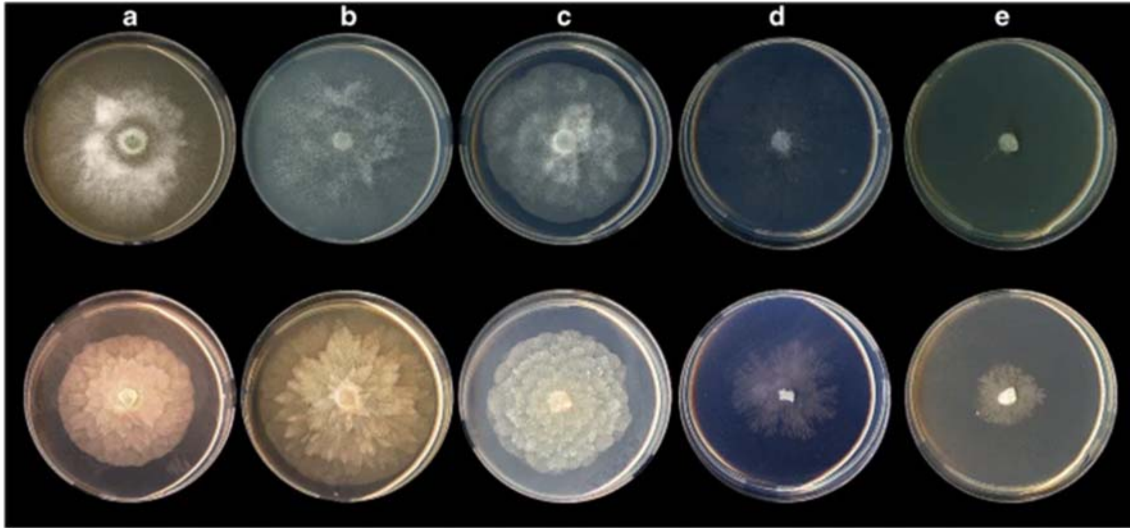

Fig. 4 Colony morphologies of *Phytophthora emzansi* sp. nov. CBS 147464 recovered in this study (top row) and *Phytophthora afrocarpa* sp. nov. CBS 147467 (bottom row) on five solid microbial culture media. **a** Carrot agar. **b** V8 agar. **c** Potato dextrose agar. **d** Corn meal agar. **e** Malt extract agar

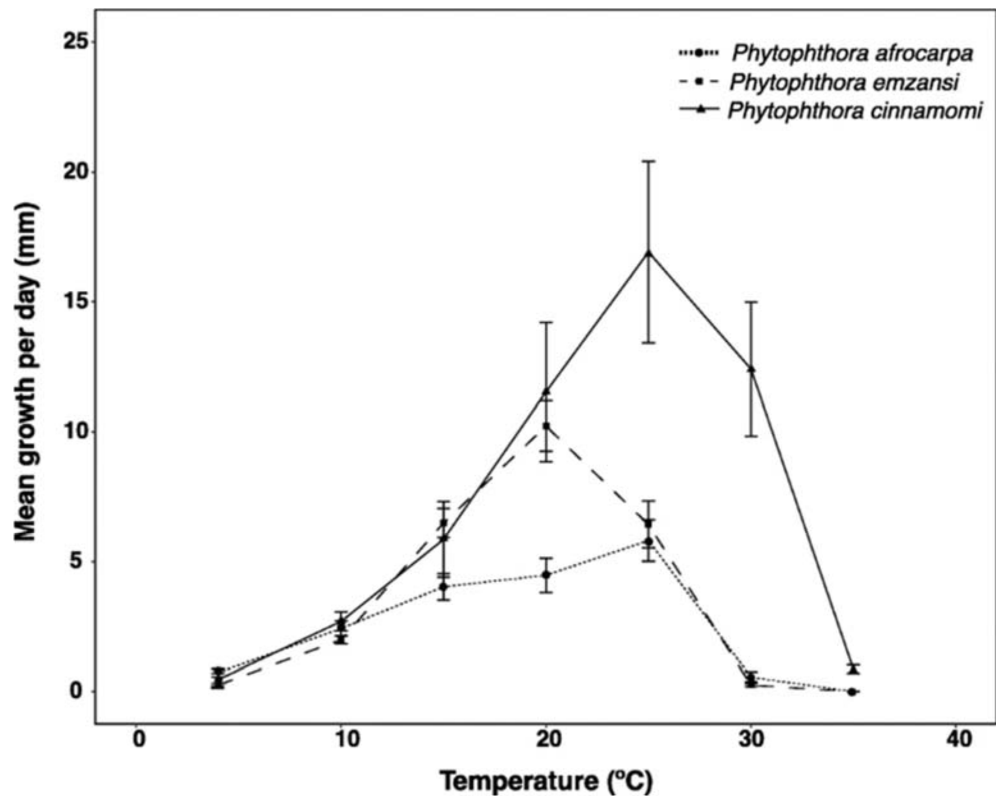

Fig. 5 Mean radial growth rates of all isolates of *Phytophthora emzansi* sp. nov. and *Phytophthora afrocarpa* sp. nov. recovered in this study on V8 agar at different temperatures. An isolate of *Phytophthora cinnamomi* (CMW48774) was used as a reference. Bars on plot points indicate standard errors

Additional material examined. SOUTH AFRICA, Western Cape Province, from Eerste River (-33.937661, 18.872178), July 2016, leg. JM Hulbert – CMW 54619 = CBS 147465; SOUTH AFRICA, Knysna Forest, Western Cape Province, from rhizosphere soil of *Afrocarpus falcatus* (-33.93149201, 22.62277904), November 2017, leg. JM Hulbert – CMW 54531 = CBS 147466; SOUTH AFRICA, Knysna Forest, Western Cape Province, from rhizosphere soil of *Afrocarpus falcatus* (-33.91666199, 22.95783539), February 2019, leg. JM Hulbert – CMW 54569; SOUTH AFRICA, Knysna Forest, Western Cape Province, from rhizosphere soil of *Rapanea melanophloeos* (-33.91666199, 22.95783539), February 2019, leg. JM Hulbert – CMW 54577; SOUTH AFRICA, Kirstenbosch National Botanical Garden, Western Cape Province, from rhizosphere soil of *Podocarpus elongata* (-33.988122, 18.428083), October 2016, leg. T Paap – CMW 50975; SOUTH AFRICA, Western Cape Province, infected plant tissue of *Agathosma betulina* (coordinates and area not indicated by the depositor), 2005, leg. K Lubbe – STE-U 6269 (=WPC P15619, CMW 56071); SOUTH AFRICA, Western Cape Province, infected plant tissue of *Agathosma betulina* (coordinates and area not indicated by the depositor), 2005, leg. K Lubbe – STE-U 6272 (=WPC P19574, CMW 56072).

Notes. The six isolates of *P. emzansi* from this study had noticeable morphological and biological differences compared to those from Bezuidenhout et al. (2010). Some of these differences are (i) sporangia shape and size, (ii) oogonia size, (iii) oospore size, (iv) position and size of antheridia, and (v) minimum and maximum growth temperatures (Table 2). This high level of variation is consistent with Bezuidenhout et al. (2010), supporting their decision not to describe this taxon. The greater number of isolates and strength of the phylogenetic inference in the present study provided sufficient confidence to describe the species.

Phytophthora emzansi and *P. capensis* are sister taxa (Bezuidenhout et al. 2010), yet they have contrasting biological and morphological characters. These include the (i) shapes and mean sizes of sporangia, (ii) sizes of oogonia, (iii) absence of abortive oogonia in *P. capensis*, (iv) oospore sizes and wall thicknesses, (v) positions and sizes of antheridia, and (vi) different in optimum growth temperatures (Table 2).

Table 2 Comparison of biological characters of between isolates of *Phytophthora emzansi* sp. nov. recovered from the present and previous studies along with *Phytophthora capensis*

	P. emzansi	***P. emzansi***	***P. capensis***
No. of isolates	6	2	3
Literature	Present study	Bezuidenhout et al. (2010)	Bezuidenhout et al. (2010)
Sporangia			
Characteristics	Solitary, occasionally in loose sympodia	Solitary, occasionally in loose sympodia	Solitary, terminal
Papillae	Semi-papillate, rarely papillate or non-papillate, often with displaced papilla	Semi-papillate	Semi-papillate
Persistence	Persistent	Persistent	Persistent
Shapes	Mostly ovoid, other shapes include elongated ovoid, pyriform, and obpyriform	Elongated wine-glass with smooth to slightly wavy sides or bent top, ovoid, sac-like shape, peanut and kidney-shaped	Limoniform, occasionally ovoid
Mean size (µm)	26.7 ± 1.01 × 23.9 ± 0.6	49.6 ± 8.6 × 27.4 ± 5.7	39.1 ± 6 x 24 ± 3.3
Range (µm)	25.0–63.8 × 17.5– 41.5	35.0-67.5 × 17.5-47.5	27.5-50 x 17.5-32.5
Proliferation	Absent	Absent	Absent
Sporangiophore attachment	Basal	Basal and basal off-set	Basal centered
L : B ratio	1.4 ± 0.02	1.8 - 1.9	1.6 - 1.7
Exit pore			
Mean size (µm)	5.6 ± 0.21	-	5.5
Range (µm)	3.8 − 11.46	5.0 - 5.7	5.0 - 7.5
Encysted zoospores (µm)	8.2 – 17.7	10.0 - 15.0	10.0 – 15.0
Sexual system	Homothallic	Homothallic	Homothallic
Oogonia			
Shape	Spherical often with tapering stalk	Spherical often with tapering stalk	Spherical without tapering stalk
Abortive oogonia	Abundant, do not develop into abnormal shapes	Abundant, often developed abnormal shapes	Not abortive or have abnormal shapes
Mean size (µm)	35.4 ± 1.05	30.7 ± 3.1	24.0 ± 2.5
Range (µm)	18.75 − 49.93	25.0 - 37.5	20.0 - 27.7
Oospore			
Characteristics	Mostly plerotic, rarely aplerotic	Plerotic and aplerotic (Ratio 53:47)	Plerotic, thick walled
Mean size (µm)	31.1 ± 0.89	27.9 ± 2.6	22.7 ± 2.0
Range (µm)	18.13 − 46.75	22.5 – 32.5	20.0 - 27.5
Wall thickness			
Mean size (µm)	2.0 ± 0.13	< 2.5	>2.5
Range (µm)	0.6−5.0	-	-
Wall index			
Mean size (µm)	0.38 ± 0.11	-	-
Range (µm)	0.45 ± 0.15	-	-
Antheridia			
Position	Amphigynous	Usually amphigynous, rarely paragynous	Paragynous
Mean Size (µm)	14.2 ± 0.8 × 16.1 ± 0.77	14.0 ± 1.4	9.01 ± 1.8
Range (µm)	6.25−24.5 × 7.8−24.9	12.5–17.5	5.0 - 12.5
Chlamydospores	Absent	Absent	Absent
Hyphal swellings	Present	Absent	Absent
Mean Size (µm)	13.4 ± 0.81	-	-

Range (µm)	3.8-28.54	-	-
Colony morphology			
Carrot agar	No distinct pattern	Loose chrysanthemum and petaloid	Chrysanthemum shape
V8 agar	No distinct pattern	-	-
Potato dextrose agar	No distinct pattern	-	-
Malt extract agar	Lax and immersed	-	-
Corn meal agar	Lax and immersed	-	-
Growth temperatures			
Minimum (ºC)	4	10	5.0
Maximum (ºC)	30	27.5	27.5
Optimum (ºC)	20	20	22.5
Lethal (ºC)	35	30	30

Phytophthora afrocarpa **T. Bose and J.M. Hulbert sp. nov.**

Mycobank. MB838532

Etymology. Name refers to the tree *Afrocarpus falcatus* (Syn. *Podocarpus falcatus*), the rhizosphere of which yielded all three isolates studied.

Type. SOUTH AFRICA, Diepwalle Forest, Western Cape Province, from rhizosphere soil of *Afrocarpus falcatus* (-33.956555, 23.152709), January 2017, leg. JM Hulbert type PREM63082, ex-type culture PPRI 28450 = CMW 54630 = CBS 147467. GenBank: ITS: MT762306, *BT*: MT762333, *COX1*: MT762315, *HSP90*: MT762324.

Description. Sporangia predominantly ovoid (84%; Figs. 6a-d), often elongated ovoid (12%; Fig. 6e), rarely obpyriform (4%), sporangia without basal septa continue to grow as hyphae (Fig. 6f), often with internal extended proliferation (Figs. 6d, g); proliferating sporangia are wine glass shaped (Fig. 6d, g); non-papillate; persistent, borne terminally on solitary sporangiophores (fig. 6a-e), abundantly produced within 48 h of adding non-sterile soil extract. Sporangia from three isolates measured (13.3) 28 ± 1.15 (49.5) × (7.4) 18.6 ± 0.74 (31.2) µm, exit pores usually wide measuring (2.9) 7.3 ± 0.66 (11.7) µm; length to breadth ratio 1.5 ± 0.008. Sporangiophores simple, attachment mostly basal centered (Figs. 6b-e), often slightly inflated at the point of attachment to the sporangia (Fig. 6e). Encysted zoospores ranging from 5.2 – 12.6 µm (Fig. 6h). Gametangia absent. Chlamydospores spherical, terminal, usually catenulate (Fig. 6i), often solitary (Fig. 6j), forming on the lateral hyphal branches (Fig. 6i), immature

chlamydospores are hyaline (Fig. 6j) turn dark brown with maturation (Fig. 6i); measuring (2.2) 26.2 ± 0.46 (36) µm in diam. Hyphae coenocytic, highly branched with hyphal swelling, often on lateral branches, globose or sub-globose in shape, measuring (4.6) 6.6 ± 0.68 (8.8) µm in diam. (Fig. 6k).

Colony morphology and growth. Slow-growing species with white, cottony filamentous hyphae having a variety of colony morphologies such as chrysanthemum shaped (CA and V8; Fig. 4a, b), stellate (PDA; Fig. 4c), lax and immersed (CMA and MEA; Fig. 4d, e). Growth extremely slow on MEA after 5 days at 20 °C in darkness (Fig. 4e). The optimum growth temperature for all three isolates on V8A was 25 °C (5.81 ± 0.4 mm/day; Fig. 5). The minimum and maximum temperatures for growth were 4 °C (0.7 ± 0.07 mm/day; Fig. 5) and 30 ºC (0.55 ± 0.09 mm/day; Fig. 5). Plates incubated at 35 °C showed no growth when tested for viability after the growth study, indicating that this temperature was lethal.

Additional material examined. SOUTH AFRICA, Diepwalle Forest, Western Cape Province, from rhizosphere soil of *Afrocarpus falcatus* (-33.956555, 23.152709), January 2017, leg. JM Hulbert – CMW 54631 = CBS 147590; SOUTH AFRICA, Diepwalle Forest, Western Cape Province, from rhizosphere soil of *Afrocarpus falcatus* (-33.956555, 23.152709), January 2017, leg. JM Hulbert – CMW 54635 = CBS 147468.

Notes. Phytophthora afrocarpa is a sister taxon to *P. gallica* and shares a few characteristic similarities, one of which is sexual sterility. This species can be distinguished from *P. gallica* by differences in (i) sporangia size, (ii) chlamydospores shape and size, (iii) colony morphologies on CA, MEA and CMA, and (iv) optimum growth temperatures (Table 3).

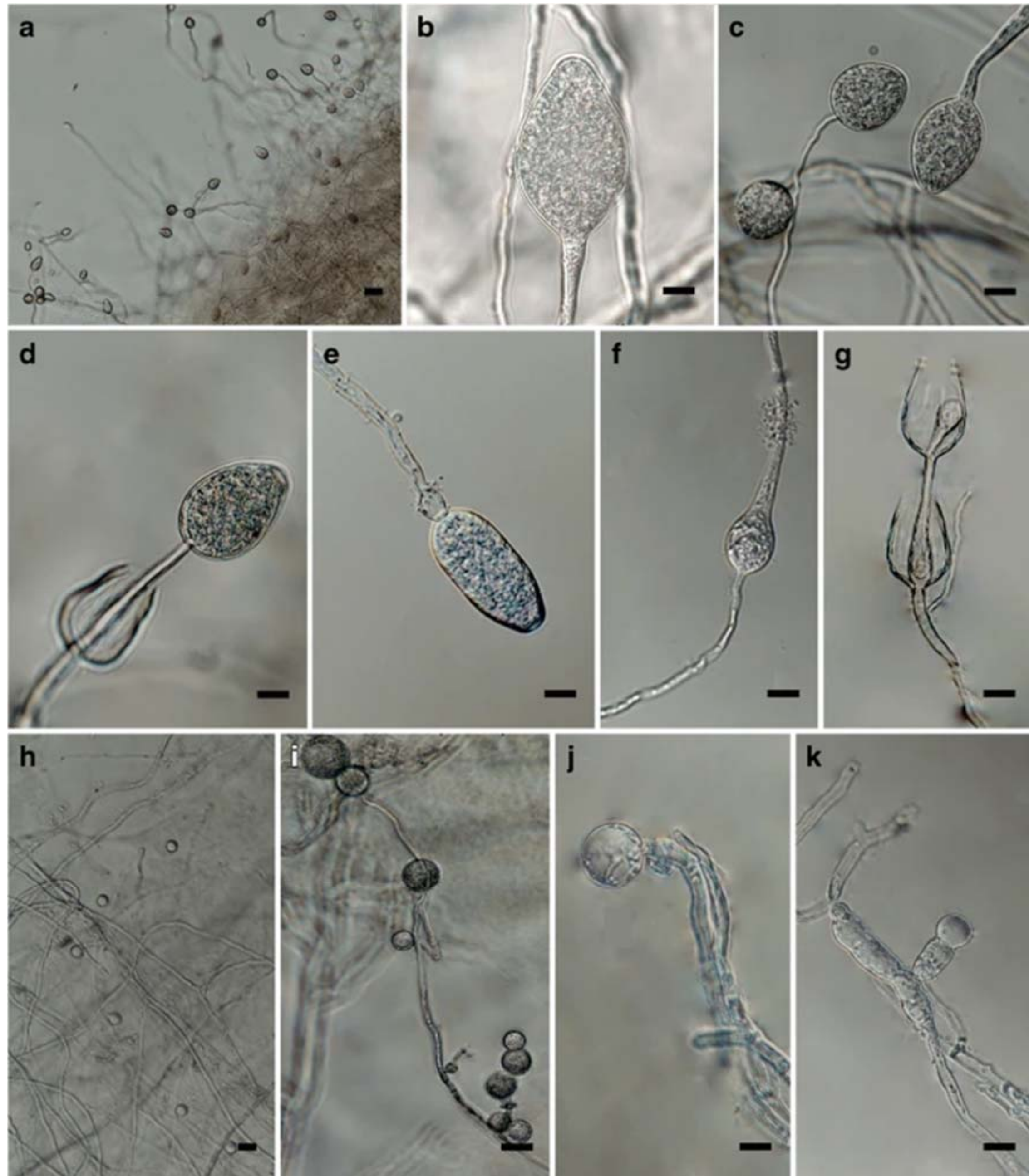

Fig. 6 Morphology of *Phytophthora afrocarpa* sp. nov. **a** various type of sporangia. **b, c** ovoid non-papillate sporangia with basally attached sporangiophore. **d** ovoid sporangia with internal extended proliferation. **e** elongated ovoid sporangium with inflated sporangial attachment. **f** sporangium without basal septum continue to grow as hyphae. **g** wine glass shaped sporangial proliferation. **h** zoospore cysts. **i** catenulate and solitary chlamydospores. **j** terminal immature hyaline chlamydospore. **k** hyphal swellings. Bars 10 µm

Table 3 Comparison of biological characters of *Phytophthora afrocarpa* sp. nov. and *Phytophthora gallica*

	P. afrocarpa	***P. gallica***
No. of isolates	3	2
Literature	Present study	Jung and Nechwatal (2008)
Clade	10b	10b
Sporangia		
Characteristics	Non-papillate, persistent	Non-papillate, persistent
Shapes	Ovoid, elongated ovoid, rarely obpyriform	Obpyriform, ovoid, peanut-like, or limoniform
Mean size (µm)	28 ± 1.15 × 18.6 ± 0.74	52.5 ± 11.0 x 27.0 ± 5.0
Range (µm)	13.3–49.5 × 7.4–31.2	30–100 × 19–48
L : B ratio	1.5 ± 0.008	2 ± 0.5
Proliferation	internal extended	internal extended or nested
Sexual system	Sterile	Sterile
Chlamydospores		
Shapes	Spherical in bunches, rarely solitary	Globose, elongated, pyriform, club-shaped, or irregular
Mean Size (µm)	26.2 ± 0.46	47 ± 6.7
Range (µm)	2.2−36	40–125 ± 5–50
Hyphal swellings	Globose or sub-globose; solitary or catenulate	Globose, sub-globose, irregular, or catenulate
Colony morphology		
CA	Chrysanthemum	No distinct pattern
V8A	Chrysanthemum	-
PDA	Stellate	-
MEA	Lax and immersed	Rosaceous
CMA	Lax and immersed	Submerged with faintly stellate with chrysanthemum margin
Growth temperatures		
Minimum (ºC)	4	5
Maximum (ºC)	30	30
Optimum (ºC)	25	20

Discussion

Nine isolates of two undescribed *Phytophthora* species from soil and water samples collected from the Greater Cape Floristic Region of South Africa emerged from this study. Six of these isolates were identified as the *Phytophthora* Clade 2 species, *P.* taxon emzansi previously recognised by Bezuidenhout et al. (2010). The remaining three isolates represented a novel taxon in *Phytophthora* Clade 10. Both DNA sequence comparisons and morphological data were used to describe these taxa as *P. emzansi* sp. nov. and *P. afrocarpa* sp. nov., respectively.

Several native and invasive *Phytophthora* species have been reported from the Greater Cape Floristic Region of South Africa (Bezuidenhout et al. 2010; von Broembsen 1984; Hulbert et al. 2019). Among these, some (*P. cinnamomi*, *P. citricola*, *P. multivora*, *P. nicotianae,* and *P. parvispora*) are widespread while others (*P. capensis*, *P. cryptogea*, and *P. drechsleri*) have limited distribution (Hulbert et al. 2019; Bezuidenhout et al. 2010; von Broembsen 1984). Isolates of *P. emzansi* recovered

in this study and those from earlier studies suggest this species is widespread in southern South Africa, where it remains associated with diverse habitats and hosts (Bezuidenhout et al. 2010; Oh et al. 2013). In contrast, even after repeated sampling, *P. afrocarpa* was exclusively isolated from Afromontane forests.

A few studies have considered the threat of *Phytophthora* species to trees in Afromontane forests. von Broembsen et al. (1986) reported the dieback of *Ocotea bullata* caused by *P. cinnamomi*. *Phytophthora capensis* was isolated from *Olea capensis* and *Curtisia dentata* (Oudemans et al. 1994; Bezuidenhout et al. 2010), but its pathogenicity to these hosts were not tested. However, Bezuidenhout et al. (2010) later examined the pathogenicity of *P. emzansi* towards seedlings of *Agathosma betulina,* a native fynbos species and found it to be non-pathogenic to this host.

The present study did not examine the pathogenicity of *P. afrocarpa* or *P. emzansi* isolates to *A. falcatus*. Even though, these *Phytophthora* species may not cause disease currently, but their impacts on hosts may alter in response to climatic change (Burgess et al. 2019; Grünwald et al. 2019; Burgess et al. 2017). Therefore, future trials should be conducted to clarify the role of these *Phytophthora* species in the Greater Cape Floristic Region, and to determine their host range and pathogenicity to species in this region.

The vegetation in southern Africa is unique and encompasses a high level of floristic diversity, which supports an enormous assortment of plant-associated microbes. Previously, a few surveys have been conducted in this area aimed at discovering novel fungi (Roets et al. 2009; Crous et al. 2006; Lee et al. 2006; Roets et al. 2014). Similarly, outcomes of the present study and that of Bezuidenhout et al. (2010) justify a need for follow-up surveys in the biomes of southern Africa, which are likely to reveal novel *Phytophthora* species.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not Applicable

Availability of data and materials

Phytophthora species described in this study are available at recognized microbial culture repositories. All sequence data generated in this study (Table 1) are available at NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/). Alignment files can be accessed via TreeBASE (http://www.treebase.org).

Conflict of interest

The authors declare that there is no conflict of interest.

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Author contributions

All authors contributed equally to the conception, writing and preparation of this manuscript. Primary isolations and preliminary identification of *Phytophthora* species were completed by Joseph M Hulbert and Trudy Paap. Morphological characteristics were measured and documented by Tanay Bose and Joseph M Hulbert. Molecular and phylogenetic analyses were completed by Tanay Bose and

Joseph M Hulbert. Culture deposition and sequence submission were completed by Tanay Bose. The first draft of the manuscript was written by Tanay Bose and all authors commented on previous versions of the manuscript. This study was supervised by Michael J Wingfield, Treena I Burgess and Francois Roets. All authors read and approved the final manuscript.

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